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Original Article

FOXP3$^+$Treg/Th17 cell imbalance in lung tissues of mice with asthma

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Abstract: Immunocyte imbalances, particularly of Th1 and Th2 type helper T (Th) cells, have been implicated in the pathogenesis of chronic inflammatory diseases like asthma. Recent studies have suggested an important role for the balance between Th17 cells and FOXP3$^+$ regulatory T cells (Treg). However, whether this balance is important in asthma remains unknown. This study sought to detect the populations of T cell subtypes (Th1, Th2, FOXP3$^+$ Treg, Th17) in lung tissue of a mouse model of asthma to understand the significance of immunocyte balances in the disease. An asthma model was generated by sensitizing ten pathogen-free BALB/c mice using a standard ovalbumin challenge; ten other mice were challenged with PBS to serve as a control group. Total white cells and differential cell counts were determined in bronchoalveolar lavage fluid, and percentages of T cell subtypes were determined using flow cytometry. The severity of inflammation in lung tissue was evaluated in tissue sections, and airway hyperresponsiveness was assessed by unrestrained plethysmography. In mice with asthma, compared to those in the control group, total white cell, eosinophil, monocyte, and lymphocyte cell counts were higher, and lung inflammation and airway hyperresponsiveness were more severe ($P<0.05$), indicating that the model of asthma was successfully generated. Further, mice with asthma had higher percentages of Th2 and Th17 cells and lower percentages of Th1 and Foxp3$^+$ Treg cells in lung tissue ($P<0.05$). Consequently, the ratios of Th1/Th2 cells and FOXP3$^+$Treg/Th17 cells were higher in the asthma group ($P<0.05$). Thus, in addition to the imbalance of Th1/Th2 cells, an imbalance of FOXP3$^+$Treg/Th17 cells may play an important role in the pathogenesis of asthma.

Keywords: Asthma, FOXP3$^+$Treg cell, Th17, ovalbumin

Introduction

Bronchial asthma is an allergic disease caused by immune dysfunction, in which helper T (Th) cells play a crucial role [1]. In particular, the imbalance of Th1 and Th2 cells is hypothesized to contribute to the pathogenesis of asthma, and is therefore becoming a focus for the treatment and prevention of asthma [2]. Further, although Th1 cells are typically thought of as anti-inflammatory, evidence indicates that they appear to aggravate allergic symptoms and asthma, and expression of the Th1 cytokine interferon (IFN)-γ correlates with antigen-induced airway hyperresponsiveness and eosinophilic infiltration [3]. Additionally, the in vitro stimulation of peripheral blood mononuclear cells from children with asthma with specific antigens, including the superantigen, house dust mite, ryegrass, and egg white, resulted in increased IFN-γ and interleukin (IL)-4 secretion; IL-4 is produced by Th2 cells, thus the finding suggests that the functions of both Th1 and Th2 cells can be enhanced in asthma [4]. A marked rise in the levels of cytokines secreted by Th1 cells has also been observed in the peripheral blood of asthma patients: a flow cytometric measurement showed that the level of IFN-γ produced by peripheral blood lymphocytes in asthma patients in remission was higher than that of patients with atopy or of healthy people [5]. Therefore, the immunological mechanisms behind asthma remain incompletely understood.

Table 1. Total white blood cells and differential cell counts in BALF of mice with and without induced asthma ($\times 10^6$ cells/L)

Group	*n*	Total white cell	Eosinophil	Monocyte	Lymphocyte
Control	10	9.56±1.21	0.04±0.01	7.62±1.15	1.89±0.74
Asthma	10	92.19±4.70	14.75±1.26	51.29±4.84	26.15±2.38
t		53.829	36.761	27.780	30.752
P		0.001	0.001	0.001	0.001

Regulatory T cells may provide further insight to the etiology of asthma. Th17 cells were recently recognized as a subtype of $CD4^+$T cells that differ from Th1/Th2 cells and are characterized by the production of IL-17. IL-17 production depends on IL-23, plays a very strong proinflammatory role, and is correlated with the etiology of multiple autoimmune diseases [6]. IL-17 mRNA and protein levels in the lung tissues, sputum, bronchoalveolar lavage fluid (BALF), and serum are significantly higher in asthma patients [7-11] and appear to be positively correlated with the degree of airway hyperresponsiveness (AHR) [12]. Therefore, Th17 cells also play an important role in asthma. Indeed, in asthma patients, Treg cells are reduced in number and function [13]. Further, $FOXP3^+$ Treg cells play an anti-inflammatory role and help maintain immunological self-tolerance [14]; they also have a mutually inhibitory relationship with Th17 cells in terms of differentiation, development, and function [15]. The $FOXP3^+$Treg/Th17 balance plays an important role in maintaining immune homeostasis; a $FOXP3^+$Treg/Th17 imbalance is correlated with the occurrence of autoimmune diseases [16]. Additional insight to these cell populations in asthma may improve the understanding of the immunological mechanism(s) of the disease.

To provide an experimental and theoretical basis for the prevention and treatment of asthma, this study analyzed the balance of $FOXP3^+$Treg/Thl7 cells in established mouse models of asthma.

Materials and methods

Experimental animals

Twenty pathogen-free female BALB/c mice (6-8 weeks old, weighing 20-22 g) were obtained from Shanghai Laboratory Animal Center (China).

Establishing mouse models of asthma

Mice were divided into an asthma group and a control group using a random number table, with 10 mice per group. To establish mouse models of asthma, the following procedure was used: On days 0 and 12, 0.2 mL of a solution containing 60 μg of ovalbumin (OVA; Sigma) with 2.25 mg of aluminum hydroxide gel (Sigma) was injected intraperitoneally to mice in the asthma group. Beginning on day 19, ultrasonic nebulization was performed with 8 mL of 5% OVA solution, 30 min daily for 5 consecutive days. In the control group, PBS was substituted for OVA to perform sensitization and nebulization.

Measurement of AHR in mice

Within 24 h after the final nebulization challenge, AHR was measured using plethysmography (Buxco Electronics, Wilmington, NC), i.e., the spontaneous breathing of mice in a conscious state was detected as described by Hamelmann et al. [17]. Briefly, mice to be measured were exposed to nebulized methacholine (USP Methapharm Inc, Brantford, Ontario, Canada) at various concentrations (3.125, 6.25, 12.5, 25, and 50 mg/mL) for 3 min, then were allowed to rest for 2 min. Next, readings were recorded for 5 consecutive min, and their mean value was obtained by mouse plethysmography software (Buxco Electronics, Wilmington, NC). AHR was expressed as the enhanced pause (Penh), which were equal to [peak expiratory pressure (PEP)/peak inspiratory pressure (PIP) × expiratory time (Te) -relaxation time (Tr)]/Tr.

Preparation of BALF and lung tissue samples

Mice were euthanized under anesthesia and fixed in the supine position. Their cervical tracheas were fully exposed, and V incisions were made at the lower ends of the tracheas. Next, 24G catheters were used to perform tracheal intubation, and 0.9 mL of normal saline solution was gently injected into each catheter. The catheter was repeatedly withdrawn three times to perform lavage for a total of 3 times (fluid recovery rate >90%). BALF was obtained for the detection of total and differential white blood cell counts as well as cytokines with flow cytom-

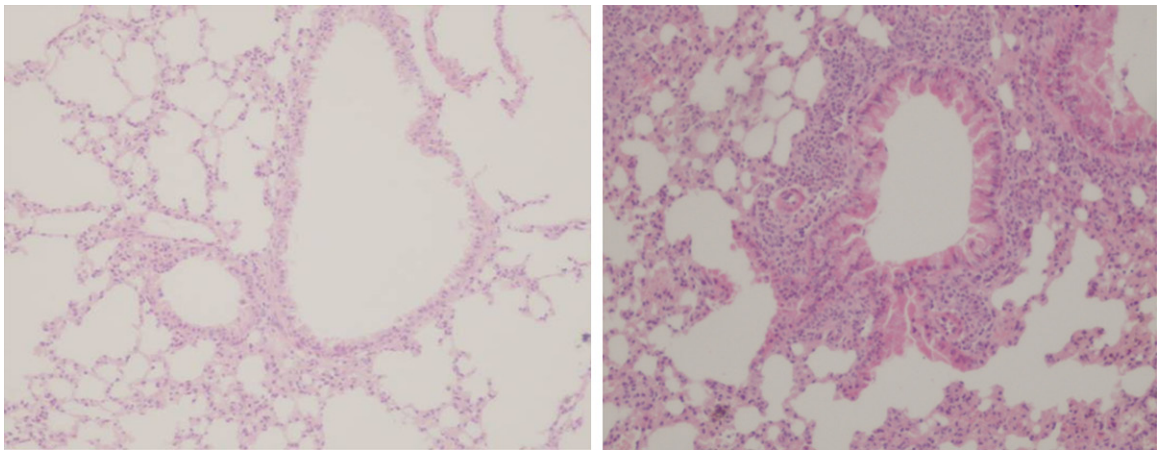

Figure 1. Inflammatory reaction of lung tissues in mice without (left) and with asthma (right) (×100).

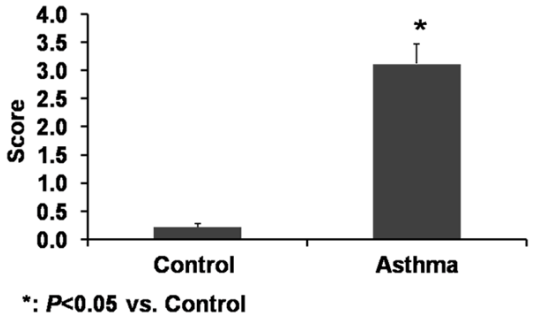

Figure 2. Histopathological score of lung tissues in mice without and with asthma. Tissue inflammation was scored as described in [18].

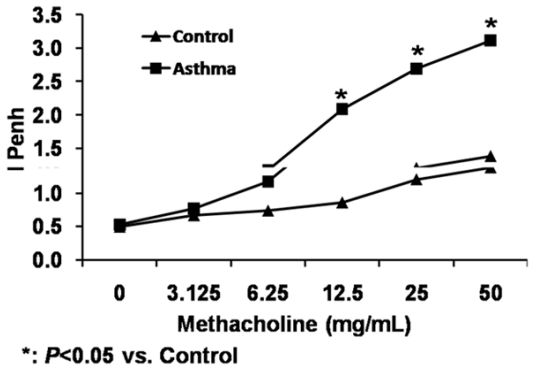

Figure 3. AHR of lung tissues in mice without and with asthma, as measured by unrestrained plethysmography.

etry. Then, the thoracic cavity of each mouse was opened and the right lung was ligated immediately. Lung tissues were collected and placed in a 10% neutral formalin solution. They were then fixed, dehydrated in an alcohol gradient, embedded in paraffin, and sectioned into 4-μm slices. Sections were stained with hematoxylin and eosin for observation under a light microscope (Olympus BX61). Scoring was performed according to a published method [18] for peribronchial inflammation, perivascular inflammation, and inflammation around pulmonary alveoli in lung tissues.

Total and differential white blood cell counts in BALF

BALF (6 μL) was drawn and allowed to fall in drops on a counting plate. Total white blood cell counts were measured (according to $N/4\times10^6$) under a microscope with an ocular magnification of 10 in a large square on one side of the plate. Remaining BALF was centrifuged (at 1500 rpm ×10 min and 4°C). Sediment was collected and smeared as well as treated with routine Wright-Giemsa stain. At least 200 cells were observed under a microscope with an ocular magnification of 40 to determine the numbers of eosinophils, monocytes, and lymphocytes.

Flow cytometry

Each mouse was perfused with PBS through the right ventricle to remove pulmonary vascular blood. Lung tissues were separated from the body, incubated for 15 min with 1 mL of PBS containing 0.002 g (0.2%) of collagenase I (Sigma-Aldrich), placed on a 300-mesh stainless steel screen, and clipped into pieces with an ophthalmic scissor. A single cell suspension was collected from the culture dish, centrifuged

Table 2. Th1, Th2, FOXP3+Treg and Th17 cell distributions (%) in mice with and without asthma

Group	*n*	Th1	Th2	Th1/Th2	Foxp3+Treg	Th17	Foxp3+Treg/Th17
Control	10	2.47±0.31	0.54±0.14	4.92±1.44	7.28±1.30	1.01±0.21	7.49±2.20
Asthma	10	1.25±0.18	0.81±0.13	1.56±0.27	5.08±1.11	1.79±0.24	2.89±0.72
t		10.733	4.646	7.237	4.082	7.639	6.283
P		0.001	0.001	0.001	0.001	0.001	0.001

at 2500 rpm for 5 min to remove the supernatant, and incubated with 2 mL of added erythrocyte lysate at room temperature for 8 min. Cells were washed twice with PBS and centrifuged to remove the supernatant, then treated with RPMI-1640 medium containing 10% FBS and 50 ng/mL PMA (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich), and 0.7 μL/mL GolgiPlug (BD Biosciences), and incubated in a 5% CO_2 incubator at 37°C for 4-6 h. Incubated cells were then collected, centrifuged to remove the supernatant, mixed to an even state, incubated in the dark at room temperature for 30 min with hamster anti-mouse CD3-Pecy7 (BD Biosciences) and rat anti-mouse CD4-FITC (BD Biosciences) antibodies. Samples were washed twice with PBS and centrifuged to remove the supernatant, then washed once with PBS and centrifuged to remove the supernatant. Following incubation at room temperature for 5 min with samples were centrifuged to remove the supernatant, and then mixed to an even state and incubated in the dark at room temperature for 30 min with rat anti-mouse IL-4-APC (BD Biosciences), rat anti-mouse IL-17-PE (BD Biosciences), and rat anti-mouse IFN-γ-PerCPcy5.5 (BD Biosciences). Samples were centrifuged to remove the supernatant and 200 μL of PBS were added with mixing. The FACSCalibur (BD Biosciences) was used for detection. To detect FOXP3+ Treg cells, samples were incubated in the dark at room temperature for 30 min with rat anti-mouse CD4-FITC (BD Biosciences) and rat anti-mouse CD25-PE (BD Biosciences). Samples were washed twice to remove the supernatant, fixed with fixative for 30 min, then washed once with PBS. Permeation fluid was applied for 5 min, and samples were incubated in the dark at room temperature for 30 min with anti-mouse FOXP3-PE-Cy5 (BD Biosciences). FACSCalibur was used to detect labeled cells.

Statistical methods

SPSS17.0 was used for the treatment of data, and measurement data are expressed as mean ± standard deviation ($\bar{\chi}$ ± s). The independent samples t-test was used to analyze and compare the detection results in various groups, wherein $P<0.05$ was considered to indicate the differences were statistically significant.

Results

Total and differential white blood cell counts in BALF

To confirm that mouse models of induced asthma displayed an immunological response, the white blood cell distributions were compared against control mice. Compared with the control group, the asthma group had a higher total white blood cell count as well as higher counts of eosinophils, monocytes, and lymphocytes in BALF ($P<0.05$; **Table 1**).

Inflammatory reaction in lung tissues of mice

To demonstrate that the inflammatory response demonstrated by white blood cell counts was reflected by lung pathology, lung tissues were compared between groups. Compared with the control group, the asthma group had a significant inflammation in the peribronchial, perivascular, and alveolar septal regions in lung tissues (**Figures 1**, **2**). The pathological score was significantly higher in the asthma group ($P<0.05$).

AHR in mice with and without induced asthma

In both groups, AHR increased with the increase of inhaled methacholine concentration, but the magnitudes of increase in AHR were different (**Figure 3**). In particular, AHR was significantly higher in the asthma group exposed to 12.5, 25, and 50 mg/mL methacholine than in the control group at the same doses ($P<0.05$).

T cell subsets in lung tissues of mice without and with asthma

To determine whether the increased inflammation and AHR demonstrated in mice with

induced asthma was reflected in altered T cell subset distributions, flow cytometry was used to detect the different subtypes of T cells. Compared with the control group, mice in the asthma group had significantly higher percentages of Th2 and Th17 cells ($CD4^+$ T cells) in lung tissues, but significantly lower percentages of Th1 and $FOXP3^+$ Treg cells ($P<0.05$; **Table 2**). Further, the Th1/Th2 and $FOXP3^+$Treg/Th17 ratios were significantly lower in mice with asthma than in controls ($P<0.05$).

Discussion

The chronic airway inflammation of asthma involves multiple types of cells. The characteristic changes of airway inflammation include inflammatory cell activation and the release of inflammatory mediators predominantly in eosinophils and lymphocytes [19]. In this study, mouse models of asthma were established with OVA challenge. In mice with induced asthma, total white blood counts and counts of eosinophils, monocytes, and lymphocytes in BALF were significantly higher than in controls, the lung tissues presented a marked inflammatory response, and airway responsiveness was significantly higher. Thus, the model of asthma presented with inflammatory cell infiltration and airway hyperresponsiveness, successfully recapitulating the inflammatory response observed in people with asthma.

The role of T helper cells in this response remains under debate and requires further investigation. Th17 cells are a relatively newly-identified Th subset differing from Th1 and Th2 cells. Th17 cells secrete IL-17, which can induce epithelial cells, endothelial cells, fibroblasts, and stromal cells to secrete IL-6, IL-8, granulocyte colony-stimulating factor, and prostaglandin E2; IL-17 can also enhance the functions of IL-1 and tumor necrosis factor (TNF) and the expression of intercellular adhesion molecule-1 (ICAM-1) on the cell surface [20]. Thus, this cytokine plays a role in promoting the inflammatory response, and, as such, is correlated with asthma [7].

$FOXP3^+$ Treg are $CD4^+$ T cells with immunosuppressive functions and are currently studied most. They have the strongest suppressive function and cover the broadest spectrum of suppression, which can prevent autoreactive T cell activation and inhibit the occurrence of autoimmune and allergic diseases, thereby playing an important role in maintaining autoimmune tolerance [21]. An imbalance of $FOXP3^+$Treg/Th17 cells can cause immune system dysfunction that contributes to the onset of autoimmune and allergic diseases, with some hypothesizing about its role in asthma [22, 23]. This study found that there were a smaller proportion of Th1 cells and $FOXP3^+$ Treg cells in lung tissues of mouse models of asthma; concurrently, the proportions of Th2 cells and Th17 cells were higher than in controls. Consequently, the Th1/Th2 ratio and the $FOXP3^+$Treg/Th17 ratio were significantly lower. These imbalances in Th1/Th2 and $FOXP3^+$Treg/Th17 cells likely participate in the pathogenesis of chronic airway inflammation in asthma; however, the specific mechanism remains unclear.

In summary, the $FOXP3^+$Treg/Th17 cell balance is altered in asthma, which suggests that dysregulation of this balance, in addition to Thl/Th2 balance, contributes to the pathogenesis of asthma. Further studies are needed to understand how this balance is disrupted and how it functions in asthma.

Disclosure of conflict of interest

None.

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